

AN ENGINEERED AND ENERGIZED FUSION PROTEIN Fv-LDP-AE WITH POTENT ANTITUMOR EFFICACY AND ANTIANGIOGENIC ACTIVITY AND USE THEREOF

FIELD OF THE INVENTION

The present invention relates to a novel antibody-based, targeted drug with anti-angiogenic and potent cancer cell killing activity – the engineered and energized fusion protein Fv-LDP-AE, its manufacturing method and use thereof in the manufacture of anti-tumor medicament.

BACKGROUND OF THE INVENTION

Matrix metalloproteinases (MMPs), especially the type IV collagenases which including MMP-2 and MMP-9, play an important role in the processes of tumor invasion and metastasis, Type IV collagenases can degrade the extracellular matrix components such as type IV collagen, destroy the integrality of basement membrane and extracellular matrix and facilitate cancer cell invasion and metastasis. In tumor the expression of type IV collagenases in endothelial cells of newly formed blood vessels is much higher than that in endothelial cells in normal tissue. The inhibition of MMPs activity can restrain invasion and metastasis as well as tumor angiogenesis. Therefore, the monoclonal antibody directed against MMP-2/MMP-9 can be used as a tumor-targeting carrier; in addition, the antibody itself can exert antitumor effect. The present invention relates to the monoclonal antibody 3G11 directed against MMP-2/MMP-9 that shows positive immunoreactivity with many kinds of cancer, and the ability of binding specifically to various human malignant tumors, especially the cancers of the digestive system.

Single-chain antibody (scFv) is the minimal, functional antibody fragment having intact antigen-binding site of an antibody by fusing VH to VL via a flexible peptide. The stability of scFv is better than that of Fv fragment. As compared with the intact antibody, scFv shows better penetration into the solid tumor. Therefore, scFv is more desirable as targeting drug carrier for solid tumor therapy.

As the highly potent “warhead” drug, lidamycin (LDM, also named C-1027 or C1027) is an enediyne-containing antibiotic produced by *Streptomyces globisporus* (accession number: CGMCC No.0135) which was isolated from a soil sample collected in Qianjiang County, Hubei Province, China. LDM is one of the macromolecular peptide antibiotics which displays the most potent cytotoxicity against tumor cells reported hitherto. In vivo experiment had demonstrated that LDM remarkably inhibited the growth of colon carcinoma 26 in mice, and also showed potent inhibitory effect on the growth of human cancer xenografts such as hepatoma Bel-7402 and cecum carcinoma Hce-8693 in nude mice (Chinese Journal of Antibiotics 1994, 19 (2):164-168).

The LDM molecule consists of two moieties, one is enediyne chromophore which is labile and is responsible for cytotoxicity; another is apoprotein (LDP) containing 110 amino acid residues which plays the role of protection for the stability of chromophore. The chromophore of LDM exists in two forms: active enediyne (AE) and inactivate chromophore. The active enediyne of LDM is extremely unstable and loses its activity in about one hour when exposed to sunlight in room temperature; namely, AE was inactivated by aromatization. AE is the active moiety of LDM, apoprotein is only responsible for protecting the AE; and the quantity of AE in LDM determines the activity of LDM. Therefore, it is essential that assuring high AE ratio in the chromophore of LDM for the bioactivity.

In the present invention, on the basis of novel constructed fusion protein Fv-LDP, the extremely potent enediyne chromophore (AE) which quality has been strictly controlled is employed to energize the fusion protein molecule, yielding an energized fusion protein Fv-LDP-AE that acts as a novel antibody-based targeting drug with better antitumor efficacy.

DETAILED DESCRIPTION OF THE INVENTION

Developing molecule down-sized antibody-targeting therapeutics with high antitumor efficacy and searching for new tumor-specific molecular targets are the main effective approaches for overcoming the barriers of antibody-based therapeutics for tumor treatment at the present time. The present inventors have constructed and attained the fusion protein of scFv and LDM with targeting function by genetic engineering technology. And the fusion protein can effectively deliver the effector

moiety to the tumor target. The protocol improves the homogeneity and efficacy of the molecule itself as compared with the immunoconjugate manufactured by chemical coupling approaches.

In one aspect, the present invention relates to an energized fusion protein Fv-LDP-AE. The fusion protein Fv-LDP consists of a single-chain Fv fragment (scFv) derived from an anti-matrix metalloproteinase MMP-2/MMP-9 monoclonal antibody 3G11, a lidamycin apoprotein LDP, a flexible protein spacer (GGGGS), and a six-histidine tag (His₆-tag).

The energized fusion protein Fv-LDP-AE composed of the fusion protein Fv-LDP and the active enediyne chromophore of lidamycin (AE, MW 843kDa).

1. Fusion protein Fv-LDP

Specifically, the gene encoding the fusion protein Fv-LDP is 1119 bp (SEQ ID NO: 1) and it encodes 372 amino acids (SEQ ID NO: 2). The molecular weight of Fv-LDP is 38.7 kDa.

An assembled fusion protein LDM-Fv had been prepared in our lab (Acta Pharmaceutica Sinica, 2000, 35<7> : 488-491). The Fv portion of that fusion protein was from different hybridoma. Said scFv (3G11) in Fv-LDP of the present invention is generated from a murine hybridoma 3D6 directed against type IV collagenase (including 92 kDa and 72 kDa). However, the scFv-M97 in LDM-Fv is from another murine hybridoma C2H5 directed to type IV collagenase (92 kDa). The sequence homology of those two scFv genes is 91%. Said gene that encodes scFv (3G11) of the present invention is 741 bp and that for scFv-M97 is 732 bp. The disparity of amino acid sequences between scFv (3G11) of the present invention and scFv-M97 is observed at 2 sites in CDR2 and 6 sites in CDR3 of the VH chain of scFv (3G11) and scFv-M97.

The differences in nucleotide sequences of those two scFv fragments lead to the change of amino acids sequences, so there exists a remarkable discrepancy in specificity and affinity for their targeted antigens between scFv (3G11) and scFv-M97.

The monoclonal antibody 3G11 of scFv (3G11) of the present invention showed a high immunological affinity for antigen MMP-2/9 as proved by Western-blotting. The monoclonal antibody 3G11 derived from hybridoma 3D6 displayed immunological

activity in various human tumor tissues (Liang Li, Xiu-jun Liu, and Yong-su Zhen. Targeted distribution and antitumor activity of the anti-type IV collagenase monoclonal antibody, Proceedings of the 2002 National Cancer Conference. 2002:202), and targeted distribution in human lung cancer xenograft transplanted into nude mice (Yao Dai, Bing Jia, Yong-su Zhen, et al. Immunoscintigraphy of anti-type IV collagenase monoclonal antibody in nude mice bearing human lung cancer xenograft, Chinese Journal of Cancer, 2003, 22(12): 1243-1248.). Moreover, the scFv (3G11) fragment retained the immunological binding activity of monoclonal antibody 3G11 of the present invention (Yong Tang, Yong-su Zhen. Expression of anti-type IV collagenase scFv fragment and inhibition of tumor cells invasion, Chinese Journal of Cancer, 2001, 20(8):801-805).

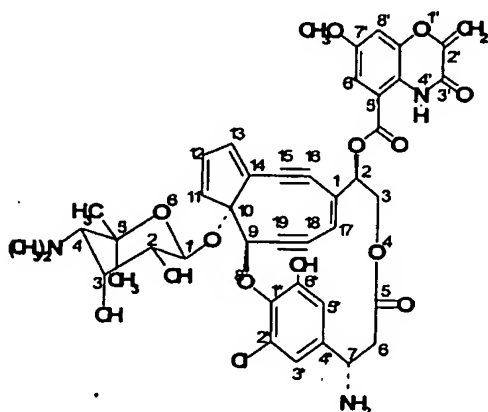
In the present invention, a flexible protein spacer (GGGGS) was inserted into the newly constructed fusion protein Fv-LDP locating between single chain scFv (3G11) and LDP, making the scFv and LDP correctly folded and formed their original conformation respectively. Therefore, both of scFv and LDP retained their own bioactivity.

2. The active enediyne chromophore, AE

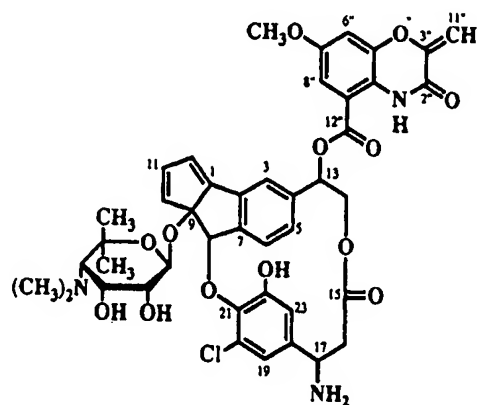
The molecular weight of LDM is 11349.1120 Dalton, which is consisted of 10505.7830 Dalton of LDP and 843.3295 Dalton of chromophore. There are two types of lidamycin chromophore. One is the active enediyne chromophore, AE, and the other is an aromatized inactivated form. The detail description on AE is displayed as below:

Chemical name of AE: (2R,7S, 9R, 10R)-7-Amino-7,8-(2'-chloro-6'-hydroxy-1',4'-phenylene)-10-(4'-deoxy-4'-dimethylamino-5',5'-dimethyl-ribosepyranosido)-4,8-dioxo-5-oxo-1,11,13-trien-15,18-diyn-tricyclo[7,7,3,0^{10,14}]-2-nondecanyl-2'',3''-dihydro-7''-methoxy-2''-methylene-3''-oxo-1'',4''-benzoxazine-5''-carboxylate

Molecular formula of AE: C₄₃ H₄₂ O₁₃ N₃ Cl



Structure of AE



Structure of aromatized chromophore

Two parts of lidamycin, LDP and chromophore, connecting with each other specially and firmly through non-covalent binding, can be dissociated and reconstituted to rebuild an energized molecule. The unique characteristic of molecular constitution, low molecular weight of AE, and potent bioactivity made lidamycin a promising “warhead” agent in constructing new monoclonal antibody targeted drugs (Xiao-yun Liu, Yong-su Zhen. Antitumor effect of lidamycin-containing monoclonal antibody immunoconjugate with downsized-molecule. *Acta Acad Med Sin*, 2001, 23 <6>: 563-567).

Another aspect of the present invention relates to the preparation of energized fusion protein Fv-LDP-AE. In details, the energized fusion protein Fv-LDP-AE was obtained by mixing active endyne chromophore of LDM (AE) with newly constructed fusion protein Fv-LDP. Depending on the specific binding of single chain antibody scFv (3G11) against MMP-2/9, the energized fusion protein Fv-LDP-AE would accumulate on the MMP-2/9 overexpressing tumor location cells and exerted its highly potent cytotoxicity against tumor. As shown, the energized fusion protein had anti-angiogenic activity and significant therapeutic effect in vivo animal experiments. It displays a promising future in cancer therapy.

The process of preparation of the energized fusion protein Fv-LDP-AE is as follows:



The preparation method of LDM can make reference to the patent (No. 00121527.2) filed on August 10, 2000 and granted by State Intellectual Property of Office of P.R.C on October of 2003. The anti-tumor activity of LDM depended on the content of AE in total chromophore of LDM which can be determined by HPLC. As set for the quality standard, the AE content should be higher than or equal to 80% of the total chromophore.

In one embodiment of the present invention, for getting the energized fusion protein Fv-LDP-AE, AE-in-methanol was added to the PBS solution (10 mM, pH 7.0) of the Fv-LDP fusion protein, with their molecular ratio of 5:1 and volume ratio of 50:1, for 12 h at room temperature and with prevention from light.

The inventor surprisingly found out that the use of high AE content chromophore

energization will result in much more potent anti-tumor activity of Fv-LDP-AE. In this invention, AE content was higher than 80%, preferably higher than 90% in LDM. In the present invention, the percentage of AE in the high quality LDM will be up to 90.63%.

In the past, the chromophore used for preparation of previously reported assembled fusion protein LDM-Fv in our lab was extracted from relatively low activity LDM prepared by old process. The AE content in total chromophore was only 60%. The IC_{50} for cancer cells by clonogenic assay was 9.5×10^{-15} M. However, in this invention, the IC_{50} value of the energized fusion protein Fv-LDP-AE was 1.658×10^{-16} M which indicated 58-fold stronger cytotoxicity against cancer cells than that of the previous assembled fusion protein LDM-Fv. In addition, as to the previous assembled fusion protein LDM-Fv, no immunobinding to human colon cancers and no *in vivo* antitumor efficacy in animal experiment were reported.

On another aspect, the present invention relates to use of said the energized fusion protein Fv-LDP-AE in preparation of medicament for the treatment of human cancers, such as colorectal carcinoma, esophagus carcinoma, gastric carcinoma, hepatocarcinoma, mammary carcinoma, ovarian carcinoma, lung carcinoma and renal carcinoma.

On another respect, the present invention relates to pharmaceutical composition comprising therapeutically effective amount of energized fusion protein Fv-LDP-AE, and optionally, said pharmaceutical composition further comprises pharmaceutical acceptable carrier and excipient compatible to the administration route and dosage thereof.

On another respect, the present invention relates to a method for treating malignant cancers, including the administration of therapeutically effective amount of said energized fusion protein or the pharmaceutical composition of the present invention to the patient with tumor.

The research of this invention indicated that the energized fusion protein Fv-LDP-AE showed selective distribution in colorectal carcinoma tissues, which overexpress type IV collagenases (MMP-2 and MMP-9). The Fv-LDP-AE displayed highly potent cytotoxicity against cancer cells, marked anti-angiogenic activity, and significant therapeutic efficacy in animal therapeutic experiments. So far, no

comparable energized fusion protein has been reported yet. Evidently, Fv-LDP-AE is one of the antibody-based fusion proteins with smallest molecule size and proved in vivo antitumor efficacy.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1. PCR amplification product and restriction enzymatic analysis of recombinant plasmids.

Lane 1, DNA MW markers; lane 2, PCR product of scFv; Line 3, PCR product of LDP; Line 4, restriction enzymatic analysis of pGEM-T-scFv/Nde I + EcoR I; Line 5, restriction enzymatic analysis of pGEM-T-LDP/EcoR I + Xho I.

Figure 2. Restriction enzymatic analysis of recombinant plasmid pEFL in recombinant strain CAMS/FLDFP.

Lane 1, DNA MW markers; lane 2, pET-30a (+); lane 3, pEFL; lane 4, pET-30a (+) /Nde I + EcoR I; lane 5, pEFL / Nde I + EcoR I; lane 6, pET-30a (+) / EcoR I + Xho I; lane 7, pEFL / EcoR I + Xho I; lane 8, pET-30a (+) / Nde I + Xho I; lane 9, pEFL / Nde I + Xho I.

Figure 3. SDS-PAGE analysis of fusion protein Fv-LDP under optimized condition.

Lane 1, standard protein MW marker; lane 2 and lane 3, total protein of *E. coli* carrying plasmid pET-30a (+) before and after IPTG induction; lane 4, and lane 5, total protein of *E. coli* carrying recombinant pEFL before and after IPTG induction; lane 6, soluble cytoplasmic fraction of *E. coli* carrying recombinant pEFL after IPTG induction; lane 7, insoluble cytoplasmic fraction of *E. coli* carrying recombinant pEFL after IPTG induction.

Figure 4. Western blotting analysis of fusion protein Fv-LDP expression with (A) anti-LDP monoclonal antibody F9 and (B) anti-His monoclonal antibody.

Lane 1, and lane 2, total protein fraction of *E. coli* carrying plasmid pET-30a (+) before and after IPTG induction; lane 3, and lane 4, total protein fraction of *E. coli* carrying recombinant pEFL before and after IPTG induction; lane 5, insoluble cytoplasmic fraction of *E. coli* carrying recombinant pEFL after IPTG induction; lane 6, soluble cytoplasmic fraction of *E. coli* carrying recombinant pEFL after IPTG induction.

Figure 5. SDS-PAGE analysis of purification of fusion protein Fv-LDP expression product using Ni-NTA affinity chromatography.

Lane 1, standard protein MW marker; lane 2 and lane 3, protein fraction before loading; lane 4, protein fraction after washing with 1x binding buffer; lane 5, protein fraction after washing with 20 mM imidazole; lane 6, 7, 8, 9, and 10, protein fraction after washing with 1 M imidazole for 1 h, 2 h, 3 h, 4 h and 5 h, respectively.

Figure 6. Separation of energized fusion protein Fv-LDP-AE from free AE on PD-10 column. PD-10 (Sephadex G-25); Eluent, 0.02 M PBS (pH 7.4); 1, Fv-LDP-AE; 2, superfluous AE.

Figure 7. Immunoreactivity of fusion protein Fv-LDP with type IV collagenase and various antigen- related cancer cells in ELISA. □, type IV collagenase; ■, HT-29 cells; ▲, H22 cells.

Figure 8. Immunohistochemical analysis of fusion protein Fv-LDP reacted with human colorectal carcinoma tissue. A, immunohistochemical staining of Fv-LDP in human colorectal carcinoma tissue; B, control, PBS instead of Fv-LDP as the primary antibody (200×, the bars represent 20 μm in length.).

Figure 9. Gelatin-zymography analysis of fusion protein Fv-LDP in HT-1080 cells. Lane 1, treated with PBS; lane 2, treated with induced product of control vector pET-30a (+); lane 3, treated with parent monoclonal antibody 3G11; lane 4, treated with fusion protein Fv-LDP (30 μM).

Figure 10. Inhibition of bFGF-stimulated angiogenesis by energized fusion protein Fv-LDP-AE in CAM assay

A, PBS control, treated with PBS alone; B, bFGF control, treated with PBS and bFGF; C, positive control, treated with LDM (0.1 μg/egg) and bFGF; D, treated with Fv-LDP-AE (0.4 μg/egg) and bFGF.

Figure 11. Cytotoxicity of energized fusion protein Fv-LDP-AE to HT-29 cells in clonogenic assay. □, Fv-LDP-AE; ■, LDM.

Figure 12. Energized fusion protein Fv-LDP-AE inhibited the growth of hepatoma 22 in mice. Mice with subcutaneously inoculated tumor cells were randomized to 6 groups: □, Control; ■, LDM (0.05 mg/kg); ▲, Fv-LDP (2.4 mg/kg); □, Fv-LDP-AE (3.2 mg/kg); □, Fv-LDP-AE (1.6 mg/kg); and □, Fv-LDP-AE (0.8 mg/kg). Mice were treated on day 1 and day 11, iv × 2. Numbers in parentheses represent the doses at mg / kg.

Figure 13. HPLC analysis of lidamycin

chromatography : Delta-PAK C4 5 μ m , 300A 150 \times 3.9 mm I.D; The fluent phase: acetonitrile (0.025%): trifluoroacetic acid (20%:80%) = 23:77; Fluent speed: 0.6 ml/min; Detection wavelength: 350 nm.

Figure 14. Mass-spectrogram of AE of lidamycin

Test apparatus : Quattro LC (MICROMASS company of England) ; Sample is dissolved by 50% acetonitrile (0.5mg/ml); the highest peak of AE: m/z is 844.4(M+1); the peak of aromatization production of AE: m/z is 846.4(M+1); The peak of isotopes of chlorine: m/z is (848M+1)

Example 1 Cloning of fusion gene encoding for the aprotein LDP of LDM and scFv (3G11) directed against MMP2/MMP9

Recombinant plasmid pCANscFv or pPIC-9kFv1027 and pIJ1027GRGDS contain scFv gene and LDP gene, respectively and were stored at our laboratory. Vector pGEM-T is from Promega Company, *E.coli* DH5 α is stored at our laboratory. PCR primer is synthesized by Saibaisheng Company with recognition sites for corresponding restriction enzymes (Takara Company) introduced therein.

Four primers were designed for obtaining scFv and LDP genes by PCR amplification.

The primers of scFv gene were the following:

PH1: 5' CGCATATG CAGGTGAAGCTGCAGCAGTCT 3'

Nde I

V_H

PL2: 5' CGGAATTC TGAACCGCCTCCACC ACGTTTGATTTCAG 3'

EcoR I

V_L

The primers of LDP gene were the following:

PLD1 : 5' CGGAATTC GCGCCCGCCTTCTCCGTCAGTCCC 3'

EcoR I

LDP

PLD2 : 5' CCGCTCGAG TCAGCCGAAGGTCAGAGCCACGTG 3'

Xho I

LDP

The heavy and light chain variable domains (V_H and V_L) of mAb 3G11 was connected by a (Gly₄Ser)₃ linker sequence to construct the recombinant single-chain Fv fragment (scFv) which has been constructed in V_H-V_L orientation using phage display technique. The cDNA coding for scFv was amplified with primers PH1 and

PL2 from the pPIC9K-Fv1027 plasmid containing scFv gene. Nde I and EcoR I restriction (underlined) sites were introduced at its 5' and 3' ends of the coding region of scFv and a cDNA fragment encoding a flexible spacer (*italics*) of the fusion protein was added to the 3' end. The LDP (apoprotein of LDM) gene fragment added with EcoR I and Xho I restriction sites (underlined) at its 5' and 3' end was obtained with primers PLD1 and PLD2 from the pIJ1027-GRGDS containing LDP gene.

PCR procedure as described below: 1. 94°C for 2 minutes; 2. 25 cycles of: 94°C for 1 minute, 55°C (scFv) or 58°C (LDP) for 1 minute, 72°C for 1 minute; and 3. 72°C for 10 minutes.

Two PCR products were purified by DNA glass-milk purification kit (BioDev), connected with pGEM-T (Promage), transfected DH5 α and screened for recombinant pGEM-T-Fv, and pGEM-T-LDP and were confirmed by enzyme digestion and DNA sequencing. scFv (3G11) gene was 741bp and coded for 247 amino acids. LDP gene was 342 bp and coded for 114 amino acids. The flexible space was 15 bp and coded for 5 amino acids. The His-tag was 18 bp and coded for 6 amino acids. The stop codon was 3 bp. The whole gene was 1119 bp and coded for 372 amino acids.

Example 2. Construction of the plasmid pEFL encoding fusion protein

The expression plasmid pET30a(+) (Invitrogen) was stored in our laboratory. The pGEM-T-Fv was digested with Nde I and EcoR I, the pGEM-T-LDP was digested with EcoR I and Xho I; and then gel-purified, respectively. Finally the two fragments were constructed into the bacterial express vector pET-30 a (+) digested with Nde I and Xho I. Then the recombinant plasmid was transfected into BL21(DE3)starTM and screened.

The recombinant expression plasmid was confirmed by the restriction endonuclease digestion (Figure 2). The fusion protein encoding construct was confirmed to be correct by DNA sequencing using two T7 primers. The pET-30 a (+) has a cluster of 6 His residues at its multi-cloning site. After translation, the His₆-Tag is available for the identification and purification of the fusion protein.

Example 3. Expression of the fusion protein Fv-LDP in *Escherichia coli* BL21 (DE3) starTM

Single colonies of the above-mentioned transformant were transplanted into LB medium containing 50 μ g/ml of kanamycin by a volume of 1:50 and grown overnight at 37°C. When the culture grown up to OD₆₀₀ = 0.9, it was induced with 0.8 mM IPTG and subsequently incubated for 4-6 hours. Cells harvested from 1 ml culture

medium by centrifugation at 12000 rpm for 1 minute were resuspended in 300 μ l PBS, lysed by sonication and then centrifuged at 12000 rpm for 10 minutes. The pellet was resuspended in 300 μ l PBS and the supernatant was analyzed for the expression of exogenous protein by 12% SDS-PAGE. The result showed that the fusion protein was presented in the inclusion bodies (Figure 3). In optimal conditions, the ratio between the fusion protein and the total bacterial protein was above 30%. Then the best expression transformant was chosen.

One transformant named CAMS/FLDFP harboring plasmid pEFL expressing fusion protein Fv-LDP was deposited at China General Microbiological Culture Collection Center of China Committee of Culture Collection of Microorganisms (Beijing) on June 24, 2003 with accession number CGMCC No.0960.

For Western blot assay, after 12% SDS-PAGE the proteins were transblotted onto a PVDF membrane under constant current about 0.65 mA/cm² for 1.5-2 hours, blocked with 5% BSA/TBS and incubated with mAb F9 against LDP or anti-His monoclonal antibody as primary antibody and then with HRP-conjugated goat anti-mouse IgG as second antibody, whereupon the membrane was visualized. It was confirmed that the CAMS/FLDFP expressed the fusion protein Fv-LDP successfully (Figure 4).

Example 4. Purification and preparation of the fusion protein Fv-LDP

The fusion protein was purified under denaturing conditions by the His-bind purification kit (Novagen). After pretreated, the Ni-NTA column was equilibrated with 3 volume of binding buffer containing 6 M urea (20 mM Tris-HCl, 0.5 M NaCl, 5 mM imidazole, pH 7.9). Then the denatured protein was loaded. The column was washed successively with: (1) 10 vol. of binding buffer (20 mM Tris-HCl, 0.5M NaCl, 5 mM imidazole, 6 M urea, pH 7.9), (2) 6 vol. of washing buffer (20 mM Tris-HCl, 0.5 M NaCl, 60 mM imidazole, 6 M urea , pH 7.9). The purified protein was finally eluted with 6 vol. of elution buffer (20 mM Tris-HCl, 0.5 M NaCl, 1 M imidazole, 6 M urea, pH 7.9) (Figure 5).

The purified protein was dialyzed for renaturation. The following steps of dialysis were taken for 12-24 hours respectively: (1) 20 mM Tris-HCl, 0.5 M NaCl, 3 M urea, 5 mM EDTA, pH8.0; (2) 20 mM Tris-HCl, 0.5 M NaCl, 1 M urea, 5 mM EDTA, 0.2 mM GSSG, 2 mM GSH, 0.4 M L-Arg, pH 8.0 and (3) 20 mM Tris-HCl, 0.5 M NaCl, 5 mM EDTA, pH 8.0. Then dialyzed in 20 mM PBS (pH 7.0) for 24 h at 4°C. The renatured protein solution was concentrated by ultrafiltration at 12000 g using Centriplus-YM or Ultrafree-MC. After desalted with PD-10 column (Sephadex G25)

the frozen-dried protein sample was stored at -80°C.

Example 5. Preparation of lidamycin and measurement of AE content

1 .Preparation of lidamycin (LDM)

The LDM-producing strain (CGMCC NO.0135) in the frozen-dried tube was resuspended in 0.7 ml salt-free water, transplanted into the Gause's NO. 1 inclined medium and grown at 28°C for about 7-10 days. Then the aerial mycelium of the strain was transferred to 100/500 ml flask containing the following medium: 1% starch, 0.5% corn syrup, 0.5% peptone, 0.5% glucose, 0.02% MgSO₄, 0.06% KI, 1.5% corn starch, 0.4% CaCO₃, pH 7.0. Shaking fermentation for 48 hours at 28°C then transferred to 1000/5000 ml flask by the volume of 5% and shacked about 18 hours at 28°C in the same medium. After that the producer was transferred to a the 200 L fermentation tank containing 100 L of medium by the volume of 2% and 0.03% defoaming agent was added. The fermentation parameters include: pressure, 0.04; temperature, 28°C; stirring speed, 400 rpm, air flow 1/1, pH 6.5-7.0, fermentation time, 96 hours. 10 L fermented liquid was centrifuged and the pH of the supernatant was adjusted to 4.0 using HCl. Then 4.5 kg of (NH₄)₂SO₄ was added, and the liquid was stirred for 3 hours at 8°C. The precipitated LDM was separated by centrifugation at 8000 rpm at 4°C for 15 min. The pellet was dissolved in 200 ml cold water and dialyzed. Then the unsolvable sediment was removed by centrifugation. The supernatant was absorbed by hydroxyl apatite column, eluted by 0.001M PBS (pH6.8) and frozen-dried. Then 1500 mg crude product dissolved in water was loaded to Sephadex G-75 column. The active part was frozen-dried. The refined LDM product with high anti-tumor activity was about 145 mg.

2. Measurement of AE content

The molecular weight of the chromophore is relatively small compared with the apoprotein LDP. In molecule constitution, AE contributes approximately 7.4% to the total LDM. Because AE is the active part of LDA and the apoprotein LDP plays a role in protection of AE, therefore, the potency of LDM preparation can be determined by the measurement of AE content in total chromophore.

The AE content was measured by HPLC. The procedure was described below. LDM sample was dissolved in the fluent phase of acetonitrile and water (23/77) was injected onto the Warters C4 half-prepared column and eluted with the elution of acetonitrile and water (23/77). The eluted liquid was auto-collected and detected by HPLC C4 analysis column.

As shown by the analysis result, the present inventor prepared LDM wherein AE composed of 90.63% of total chromophore (Fig.13). The product satisfied with the quality standard of LDM. It was a refined product with high AE content. Finally, the frozen-dried sample was stored at -80°C for the succeeding usage.

As shown by Quattro LC quadrupole mass spectrometry (Fig. 14), AE peak was 844.4 (m+1) and its molecular weight was 843 Da. The molecular weight of inactive aromatized form of chromophore was 845 Da.

Because AE was unstable and would lose activity in one hour, all the above processes were performed at 4°C protecting from illumination and finished as soon as possible in order to reduce the production of inactive chromophore.

Example 6. Preparation of the energized fusion protein Fv-LDP-AE

After measurement of relative amount of AE, 10 mg refined frozen-dried LDM were added into 5 ml cold methanol and shaken for 1 hour at -20°C preventing from illumination, shake one more time during the course, then centrifuged at 12000 rpm for 20 min. The supernatant was rich in AE and the sediment contained the apoprotein. The extraction process repeated two times. Through the methanol vaporized naturally, AE was concentrated at 4°C, preventing from illumination. Fv-LDP was diluted in 0.01 mol/l PBS (pH 7.0), and 5 times of AE by molecular ratio in-methanol were added to the Fv-LDP-containing PBS solution in the volume ratio of 1:50. After shaking, the mixture was placed at room temperature for 12 hours. Then, separation and purification were performed using PD-10 (Sephadex G-25 column, Pharmacia) and detected by A280 nm. The excessive, unbinding AE was removed and the energized fusion protein Fv-LDP-AE was collected, super-filtrated and concentrated, and frozen-dried. Finally, the frozen-dried sample of Fv-LDP-AE was stored at -80°C for later use (Figure 6).

Example 7. Immunoreactivity of the fusion protein Fv-LDP

Immunoreactivity of the fusion protein Fv-LDP was detected by indirect ELISA. 10 µg/ml type-IV collagenase (diluted by 0.05 M PBS, pH 7.4) were coated to 96-well ELISA plates as antigen at 4°C overnight, 100 µl for each well. HT-1080 cells or HT-29 cells were seeded at 10⁴/well in 96-well plates at 37°C overnight. After removing the supernatant, cells were fixed by 0.25% glutaraldehyde as cell antigen. By blocking with 100 µl of 10% skim milk, then 100 µl of serial concentrations of fusion protein were added into each well, and incubated for 1 hour at 37°C. After

washing, plates were incubated with 100 μ l of 1 μ g/ml anti-LDP mAb F9 and goat-anti-mouse HRP-IgG for 1 hour at 37°C, respectively. After washing 3~4 times, 100 μ l of O-phenylenediamine-hydrogen peroxide (OPD-H₂O₂) as a chromogen for visualized reaction was added. The absorbance readings (490 nm) were taken using a Microplate Reader. The binding activities of fusion protein Fv-LDP prepared from example 4 to type-IV collagenase, HT-1080 cells, and HT-29 cells, respectively, were all positive (Figure 7).

Example 8. Immunoreactivity of fusion protein Fv-LDP with the human tumor tissues

For characterizing the immunoreactivity of Fv-LDP with human colorectal carcinoma, immunohistochemical (IHC) staining was performed by the labeled streptavidin-biotin immunoperoxidase technique with SABC Kit. The sections were blocked by normal goat serum for 20 min. The excessive liquid was removed. The sections without washing were added with diluted Fv-LDP and incubated in room temperature; then mAb F9 and biotinylated goat-anti-mouse IgG were added. Finally SABC reagent was added. DAB kit was used as a chromogen for visualized reaction at room temperature. The sections were counter-stained by hematoxylin, dehydrated, sealed, and observed with microscope.

The results showed that Fv-LDP was positively stained with the MMP-2/MMP-9 in the sections of human colon adenocarcinoma by immunohistochemical staining. The positively stained particles were located in plasma of cancer cells of colon adenocarcinoma (Figure 8).

Example 9. Inhibition of secretion of type-IV collagenase in cancer cells treated with fusion protein Fv-LDP

Exponentially growing HT-1080 cells were seeded at 10⁵ cells/ml each well in 24-well plates and incubated at 37°C, 5%CO₂. After 24 hours, the culture medium was removed and 200 μ l serum-free RPMI 1640 medium (Roswell Park Memorial institute 1640) were added, further incubated for 2 hours. Then, the cells were treated with 100 μ l of Fv-LDP each well at 37°C for 24 hours. After culture medium harvesting, it was centrifuged at 500 g to remove cellular debris, the concentration of protein in the medium was measured by Bradford method. All sample volumes were adjusted according to the concentration and then separated by non-denaturalization electrophoresis on SDS-PAGE. Thereafter, gels were washed with 2.5% Triton-X 100 for 30 min. Gels were then incubated for 16 hours at 37°C in the reaction buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 10 mM CaCl₂, 1 μ M ZnCl₂) and stained with

0.1% Coomassie-brilliant blue R-250. The location of gelatinolytic activity is detectable as a clear band in the background of uniform staining.

The result showed that the secretion of type IV collagenase in HT-1080 cells was significantly reduced by fusion protein Fv-LDP (Figure 9).

Example 10. Anti-angiogenic activity of the energized fusion protein Fv-LDP-AE

Anti-angiogenic activity of Fv-LDP-AE was examined in chick embryoallantoic membrane (CAM) assay. The surface of 7-day-old post-fertilization chick eggs (White Leghorn) in a 60% humidified incubator at 37°C was sterilized and the CAM was exposed by cutting a window (2 cm²) on the egg shell using the false air-sac technique. After 24 h, the growth factor (bFGF) at dose of 200 ng/egg was dipply added to agarose disks with the energized fusion protein Fv-LDP-AE which was prepared as mentioned in Example 6, and then the disks were placed on top of the CAM. After the windows were sealed with transparent tape, the eggs were incubated for further 48 h. The results shown in Figure 10 indicated that Fv-LDP-AE significantly suppress angiogenesis stimulated by bFGF.

Example 11. Cytotoxicity of the energized fusion protein Fv-LDP-AE to cancer cells

Cytotoxicity of the energized fusion protein Fv-LDP-AE to cancer cells is measured by clonogenic assay. HT-29 cells of exponential growth were seeded at concentration of 50 cells/0.2ml/well in 96-well plates and incubated for 24 hours. Then 50 µl of various concentrations of Fv-LDP-AE were added and further incubated at 37°C for 1 hour. Triplicate wells were designed for each concentration. After washing the wells two times with serum-free RPMI 1640 medium, cells were continuously incubated for 7 days. Colony number were counted under microscopy on day 7. The result indicated Fv-LDP-AE prepared from Example 6 displayed extremely potent cytotoxicity to cancer cells. The IC₅₀ value of Fv-LDP-AE for HT-29 cells was 1.65×10⁻¹⁶ M (Figure 11).

Example 12. *In vivo* therapy studies of the energized fusion protein Fv-LDP-AE.

Sixty KM mice, body weight ranging between 18-22 g were randomly divided into 6 groups, 10 mice each group. On day 0, hepatoma 22 cells (1.5×10⁶ cells/0.2ml/mouse) were transplanted subcutaneously into the right axilla of mice.

After 24 hours, the tumor bearing mice were treated by injection of Fv-LDP, free LDM and three doses Fv-LDP-AE into tail vein twice, on day 1 and day 10 respectively. Mice of the control group were injected with saline. Diameter of the tumor was measured every 3-4 days during the experiment. Evaluation of tumor volume was followed the formula: $V(\text{cm}^3) = 1/2ab^2$ (a, long diameter; b, short diameter of tumor.)

The results on the day 21 of the *in vivo* experiment indicated that the energized fusion protein Fv-LDP-AE as prepared in Example 6 significantly inhibited the growth of H22 tumors at 0.8 mg/kg, 1.6mg/kg, and 3.2 mg/kg in a dose dependent manner (Table 1).

Table 1. Inhibition of the growth of hepatoma 22 by energized fusion protein Fv-LDP-AE in mice

Groups	Doses (mg/kg)	Mice number begin/end	BWC (g)	Tumor volume (cm ³) $\bar{x} \pm \text{SD}$	Inhibition rate (%)
Control	-	10/10	+22	14.6±4.3	
LDM	0.05	10/10	+19.5	4.3±2.6	70.3*
Fv-LDP	2.4	10/10	+15.7	11.9±5.8	18.7
Fv-LDP-AE	3.2	10/10	+4.5	0.6±0.3	95.9* [▲]
Fv-LDP-AE	1.6	10/10	+8.7	1.8±1.2	87.8* [▲]
Fv-LDP-AE	0.8	10/10	+9.7	2.1±1.1	85.7* [▲]

$P < 0.01$ vs. control, indicated by *, and $P < 0.05$ vs. LDM, indicated by [▲].

As shown in the growth curve (Figure 12), the highest efficacy of the Fv-LDP-AE was observed at tolerated dose of 3.2 mg/kg with the inhibition rates of 92.2%, 95.2% and 95.9% on day 14, day 17, and day 21, respectively. No body weight loss and other severe side-effects were found during the experiment. This indicates that mice well tolerated the doses.